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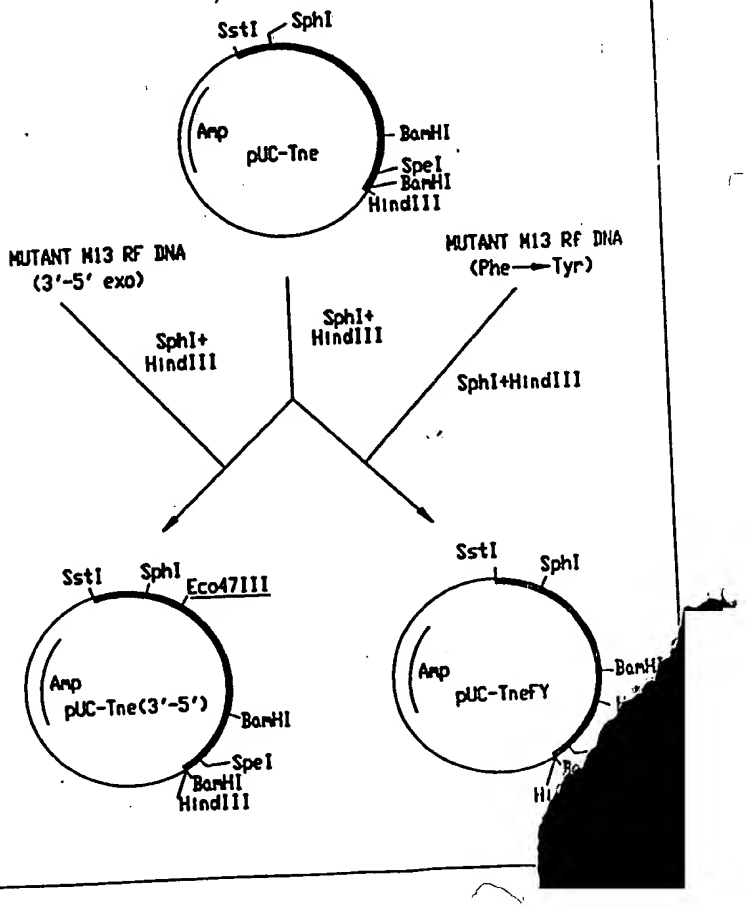
(54) Title: CLONED DNA POLYMERASES FROM THERMOTOGA NEAPOLITANA AND MUTANTS THEREOF

(57) Abstract

The invention relates to a substantially pure thermostable DNA polymerase from *Thermotoga neapolitana* (Tne) and mutants thereof. The Tne DNA polymerase has a molecular weight of about 100 kilodaltons and is more thermostable than Taq DNA polymerase. The mutant Tne DNA polymerase has at least one mutation selected from the group consisting of (1) a first mutation that substantially reduces or eliminates 3' → 5' exonuclease activity of said DNA polymerase; (2) a second mutation that substantially reduces or liminates 5' → 3' exonuclease activity of said DNA polymerase; (3) a third mutation in the O helix of said DNA polymerase resulting in said DNA polymerase becoming non-discriminating against dideoxynucleotides. The present invention also relates to the cloning and expression of the wild type or mutant Tne DNA polymerase in *E. coli*, to DNA molecules containing the cloned gene, and to host cells which express said genes. The Tne DNA polymerase of the invention may be used in well-known DNA sequencing and amplification reactions.



Background Information

DNA polymerases synthesize the formation of DNA molecules which are complementary to a DNA template. Upon hybridization of a primer to the single-stranded DNA template, polymerases synthesize DNA in the 5′ to 3′ direction, successively adding nucleotides to the 3′-hydroxyl group of the growing strand. Thus, in the presence of deoxyribonucleoside triphosphates (dNTPs) and a primer, a new DNA molecule, complementary to the single stranded DNA template, can be synthesized.

A number of DNA polymerases have been isolated from mesophilic microorganisms such as *E. coli*. A number of these mesophilic DNA polymerases have also been cloned. Lin *et al.* cloned and expressed T4 DNA polymerase in *E. coli* (*Proc. Natl. Acad. Sci. USA 84*:7000-7004 (1987)). Tabor *et al.* (U.S. Patent No. 4,795,699) describes a cloned T7 DNA polymerase, while Minkley *et al.* (*J. Biol. Chem. 259*:10386-10392 (1984)) and Chatterjee (U.S. Patent No. 5,047,342) described *E. coli* DNA polymerase I and the cloning of T5 DNA polymerase, respectively.

Although DNA polymerases from thermophiles are known, relatively little investigation has been done to isolate and even clone these enzymes. Chien *et al.*, *J. Bacteriol. 127*:1550-1557 (1976) describe a purification scheme for obtaining a polymerase from *Thermus aquaticus* (Taq). The resulting protein had a molecular weight of about 63,000 daltons by gel filtration analysis and 68,000 daltons by sucrose gradient centrifugation. Kaledin *et al.*, *Biokhymiya* 45:644-51 (1980) disclosed a purification procedure for isolating DNA polymerase from *T. aquaticus* YT1 strain. The purified enzyme was reported to be a 62,000 dalton monomeric protein. Gelfand *et al.* (U.S. Patent No. 4,889,818) cloned a gene encoding a thermostable DNA polymerase from *Thermus aquaticus*. The molecular weight of this protein was found to be about 86,000 to 90,000 daltons.

Simpson *et al.* purified and partially characterized a thermostable DNA polymerase from a *Thermotoga* species (*Biochem. Cell. Biol. 86*:1292-1296 (1990)). The purified DNA polymerase isolated by Simpson *et al.* exhibited a

5'-exonuclease activity (Joyce *et. al., J. Biol. Chem. 257*:1958-64 (1990).) Polymerases lacking this activity are useful for DNA sequencing.

Most DNA polymerases also contain a 3'→5' exonuclease activity. This exonuclease activity provides a proofreading ability to the DNA polymerase. A T5 DNA polymerase that lacks 3'→5' exonuclease activity is disclosed in U.S. Patent No. 5,270,179. Polymerases lacking this activity are useful for DNA sequencing.

The polymerase active site, including the dNTP binding domain is usually present at the carboxyl terminal region of the polymerase (Ollis *et al., Nature 313*:762-766 (1985); Freemont *et al., Proteins 1*:66-73 (1986)). It has been shown that Phe^{762} of *E. coli* polymerase I is one of the amino acids that directly interacts with the nucleotides (Joyce & Steitz, *Ann. Rev. Biochem. 63*:777-822 (1994); Astatke, *J. Biol. Chem. 270*:1945-54 (1995)). Converting this amino acid to a Tyr results in a mutant DNA polymerase that does not discriminate against dideoxynucleotides and is highly processive. See copending U.S. Application No. 08/525,087, of Deb K. Chatterjee, filed September 8, 1995, entitled "Mutant DNA Polymerases and the Use Thereof," which is expressly incorporated herein by reference.

Thus, there exists a need in the art to develop thermostable processive DNA polymerases. There also exists a need in the art to obtain wild type or mutant DNA polymerases that are devoid of exonuclease activities and are non-discriminating against dideoxynucleotides.

Summary of the Invention

The present invention satisfies these needs in the art by providing additional DNA polymerases useful in molecular biology. Specifically, this invention includes a thermostable DNA polymerase having a molecular weight of about 100 kilodaltons. More specifically, the DNA polymerase of the invention is isolated from *Thermotoga neapolitana* (Tne). The *Thermotoga* species preferred for isolating the DNA polymerase of the present invention was isolated

from an African continental solfataric spring (Windberger *et al.*, *Arch. Microbiol. 151.* 506-512, (1989)).

The Tne DNA polymerase of the present invention is extremely thermostable, showing more than 50% of activity after being heated for 60 minutes at 90°C with or without detergent. Thus, the DNA polymerase of the present invention is more thermostable than Taq DNA polymerase.

The present invention is also directed to cloning a gene encoding a *Thermotoga neapolitana* DNA polymerase enzyme. DNA molecules containing the Tne DNA polymerase gene, according to the present invention, can be transformed and expressed in a host cell to produce a Tne DNA polymerase having a molecular weight of 100 kilodaltons. Any number of hosts may be used to express the *Thermotoga* DNA polymerase gene of the present invention; including prokaryotic and eukaryotic cells. Preferably, prokaryotic cells are used to express the DNA polymerase of the invention. The preferred prokaryotic hosts according to the present invention is *E. coli*.

The Tne DNA polymerase of the invention may be used in well known DNA sequencing (dideoxy DNA sequencing, cycle DNA sequencing of plasmid DNAs, etc.) and DNA amplification reactions.

The present invention is also directed to mutant thermostable DNA polymerases. More specifically, the mutant DNA polymerases of the invention are derived from *Thermotoga neapolitana* and are substantially reduced or devoid of 3′→5′ exonuclease activity, 5′→3′ exonuclease activity, or is nondiscriminating against dideoxynucleotides. The present invention also relates to mutants having more than one of these properties, and DNA molecules containing the mutant Tne DNA polymerase enzyme genes. These mutants may also be used in well known DNA sequencing and DNA amplification reactions.

Detailed Description of the Preferred Embodiments

Definitions

In the description that follows, a number of terms used in recombinant DNA technology are extensively utilized. In order to provide a clearer and consistent understanding of the specification and claims, including the scope to be given such terms, the following definitions are provided.

Cloning vector. A plasmid, cosmid or phage DNA or other DNA molecule which is able to replicate autonomously in a host cell, and which is characterized by one or a small number of endonuclease recognition sites at which such DNA sequences may be cut in a determinable fashion without loss of an essential biological function of the vector, and into which DNA may be spliced in order to bring about its replication and cloning. The cloning vector may further contain a marker suitable for use in the identification of cells transformed with the cloning vector. Markers, for example, are tetracycline resistance or ampicillin resistance.

Expression vector. A vector similar to a cloning vector but which is capable of enhancing the expression of a gene which has been cloned into it, after transformation into a host. The cloned gene is usually placed under the control of (i.e., operably linked to) certain control sequences such as promoter sequences.

Recombinant host. Any prokaryotic or eukaryotic or microorganism which contains the desired cloned genes in an expression vector, cloning vector or any DNA molecule. The term "recombinant host" is also meant to include those host cells which have been genetically engineered to contain the desired gene on the host chromosome or genome.

Host. Any prokaryotic or eukaryotic microorganism that is the recipient of a replicable expression vector, cloning vector or any DNA molecule. The DNA molecule may contain, but is not limited to, a structural gene, a promoter and/or an origin of replication.

complementary to said template or a portion thereof. The newly synthesized DNA molecule, according to the invention, may be equal or shorter in length than the original DNA template. Mismatch incorporation during the synthesis or extension of the newly synthesized DNA molecule may result in one or a number of mismatched base pairs. Thus, the synthesized DNA molecule need not be exactly complementary to the DNA template.

Incorporating. The term "incorporating" as used herein means becoming a part of a DNA molecule or primer.

Amplification. As used herein "amplification" refers to any *in vitro* method for increasing the number of copies of a nucleotide sequence with the use of a DNA polymerase. Nucleic acid amplification results in the incorporation of nucleotides into a DNA molecule or primer thereby forming a new DNA molecule complementary to a DNA template. The formed DNA molecule and its template can be used as templates to synthesize additional DNA molecules. As used herein, one amplification reaction may consist of many rounds of DNA replication. DNA amplification reactions include, for example, polymerase chain reactions (PCR). One PCR reaction may consist of 30 to 100 "cycles" of denaturation and synthesis of a DNA molecule.

Oligonucleotide. "Oligonucleotide" refers to a synthetic or natural molecule comprising a covalently linked sequence of nucleotides which are joined by a phosphodiester bond between the 3′ position of the pentose of one nucleotide and the 5′ position of the pentose of the adjacent nucleotide.

Nucleotide. As used herein "nucleotide" refers to a base-sugar-phosphate combination. Nucleotides are monomeric units of a nucleic acid sequence (DNA and RNA). The term nucleotide includes deoxyribonucleoside triphosphates such as dATP, dCTP, dUTP, dGTP, dTTP, or derivatives thereof. Such derivatives include, for example, dm, [αS]dATP and 7-deaza-dGTP. The term nucleotide as used herein also refers to dideoxyribonucleoside triphosphates (ddNTPs) and their derivatives. Illustrated examples of dideoxyribonucleoside triphosphates include, but are not limited to, ddATP, ddCTP, ddGTP, ddITP, and ddTTP. According to the present invention, a "nucleotide" may be unlabeled or detectably labeled by

A "DNA polymerase substantially reduced in 3′-to-5′ exonuclease activity" is defined herein as either (1) a mutated DNA polymerase that has about or less than 10%, or preferably about or less than 1%, of the 3′-to-5′ exonuclease activity of the corresponding unmutated, wild-type enzyme, or (2) a DNA polymerase having a 3′-to-5′ exonuclease specific activity which is less than about 1 unit/mg protein, or preferably about or less than 0.1 units/mg protein. A unit of activity of 3′-to-5′ exonuclease is defined as the amount of activity that solubilizes 10 nmoles of substrate ends in 60 min. at 37°C, assayed as described in the "BRL 1989 Catalogue & Reference Guide", page 5, with *Hha*I fragments of *lambda* DNA 3′-end labeled with [^{3}H]dTTP by terminal deoxynucleotidyl transferase (TdT). Protein is measured by the method of Bradford, *Anal. Biochem. 72*:248 (1976). As a means of comparison, natural, wild-type T5-DNAP or T5-DNAP encoded by pTTQ19-T5-2 has a specific activity of about 10 units/mg protein while the DNA polymerase encoded by pTTQ19-T5-2(Exo$^-$) (U.S. 5,270,179) has a specific activity of about 0.0001 units/mg protein, or 0.001% of the specific activity of the unmodified enzyme, a 10^5-fold reduction.

A "DNA polymerase substantially reduced in 5′-to-3′ exonuclease activity" is defined herein as either (1) a mutated DNA polymerase that has about or less than 10%, or preferably about or less than 1%, of the 5′-to-3′ exonuclease activity of the corresponding unmutated, wild-type enzyme, or (2) a DNA polymerase having 5′-to-3′ exonuclease specific activity which is less than about 1 unit mg protein, or preferably about or less than 0.1 units/mg protein.

Both of these activities, 3'-to-5' exonuclease activity and 5'-to-3' exonuclease activity, can be observed on sequencing gels. Active 5'-to-3' exonuclease activity will produce nonspecific ladders in a sequencing gel by removing nucleotides from growing primers. 3'-to-5' exonuclease activity can be measured by following the degradation of radiolabeled primers in a sequencing gel. Thus, the relative amounts of these activities, e.g. by comparing wild-type and mutant polymerases, can be determined from these characteristics of the sequencing gel.

Igaki, (*Jpn. J. Bacteriol. 33*:729-742 (1978)). Broad-host range plasmids or cosmids, such as pCP13 (Darzins and Chakrabarbary, *J. Bacteriol. 159*:9-18, 1984) can also be used for the present invention. The preferred vectors for cloning the genes of the present invention are prokaryotic vectors. Preferably, pCP13 and pUC vectors are used to clone the genes of the present invention.

The preferred host for cloning the wild type or mutant DNA polymerase genes of the invention is a prokaryotic host. The most preferred prokaryotic host is *E. coli*. However, the wild type or mutant DNA polymerase genes of the present invention may be cloned in other prokaryotic hosts including, but not limited to, *Escherichia, Bacillus, Streptomyces, Pseudomonas, Salmonella, Serratia*, and *Proteus*. Bacterial hosts of particular interest include *E. coli* DH10B, which may be obtained from Life Technologies, Inc. (LTI) (Gaithersburg, MD).

Eukaryotic hosts for cloning and expression of the wild type or mutant DNA polymerases of the present invention include yeast, fungi, and mammalian cells. Expression of the desired DNA polymerase in such eukaryotic cells may require the use of eukaryotic regulatory regions which include eukaryotic promoters. Cloning and expressing the wild type or mutant DNA polymerase gene of the invention in eukaryotic cells may be accomplished by well known techniques using well known eukaryotic vector systems.

Once a DNA library has been constructed in a particular vector, an appropriate host is transformed by well known techniques. Transformed colonies are plated at a density of approximately 200-300 colonies per petri dish. Colonies are then screened for the expression of a heat stable DNA polymerase by transferring transformed *E. coli* colonies to nitrocellulose membranes. After the transferred cells are grown on nitrocellulose (approximately 12 hours), the cells are lysed by standard techniques, and the membranes are then treated at 95°C for 5 minutes to inactivate the endogenous *E. coli* enzyme. Other temperatures may be used to inactivate the host polymerases depending on the host used and the temperature stability of the DNA polymerase to be cloned. Stable DNA polymerase activity is then detected by assaying for the presence of DNA

nucleotides encoding the 219 amino terminal amino acids of Tne DNA polymerase. Examples of such a clone are pTTQTne535FY and pTTQTne5FY.

Tne DNA polymerase mutants can also be made to render the polymerase non-discriminating against non-natural nucleotides such as dideoxynucleotides. By way of example, one Tne DNA polymerase mutant having this property substitutes a Tyr for Phe at amino acid 67 as numbered in Figure 5. Other changes within the O helix of various polymerases such as other point mutations, deletions, and insertions can also be made.

B. Enhancing Expression of Thermotoga neapolitana DNA Polymerase

To optimize expression of the wild type or mutant *Thermotoga* DNA polymerases of the present invention, inducible or constitutive promoters are well known and may be used to express high levels of a polymerase structural gene in a recombinant host. Similarly, high copy number vectors, well known in the art, may be used to achieve high levels of expression. Vectors having an inducible high copy number may also be useful to enhance expression of *Thermotoga* DNA polymerase in a recombinant host.

To express the desired structural gene in a prokaryotic cell (such as, *E. coli, B. subtilis, Pseudomonas*, etc.), it is necessary to operably link the desired structural gene to a functional prokaryotic promoter. However, the natural *Thermotoga neapolitana* promoter may function in prokaryotic hosts allowing expression of the polymerase gene. Thus, the natural *Thermotoga* promoter or other promoters may be used to express the DNA polymerase gene. Such other promoters may be used to enhance expression and may either be constitutive or regulatable (i.e., inducible or derepressible) promoters. Examples of constitutive promoters include the *int* promoter of bacteriophage λ, and the *bla* promoter of the β-*lactamase* gene of pBR322. Examples of inducible prokaryotic promoters include the major right and left promoters of bacteriophage λ (P_L and P_R), *trp*, *recA*, *lacZ*, *lacI*, *gal*, *trc*, and *tac* promoters of *E. coli*. The *B. subtilis* promoters include α-amylase (Ulmanen *et al.*, *J. Bacteriol* 162:176-182 (1985)) and *Bacillus*

(1986). Media formulations are also described in DSM or ATCC Catalogs and Sambrook *et al.*, In: *Molecular Cloning, A Laboratory Manual* (2nd ed.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989).

Thermotoga neapolitana and recombinant host cells producing the DNA polymerase of this invention can be separated from liquid culture, for example, by centrifugation. In general, the collected microbial cells are dispersed in a suitable buffer, and then broken down by ultrasonic treatment or by other well known procedures to allow extraction of the enzymes by the buffer solution. After removal of cell debris by ultracentrifugation or centrifugation, the DNA polymerase can be purified by standard protein purification techniques such as extraction, precipitation, chromatography, affinity chromatography, electrophoresis or the like. Assays to detect the presence of the DNA polymerase during purification are well known in the art and can be used during conventional biochemical purification methods to determine the presence of these enzymes.

D. Uses of Thermotoga neapolitana DNA polymerase

The wild type and mutant *Thermotoga neapolitana* DNA polymerases (Tne) of the present invention may be used in well known DNA sequencing, DNA labeling, and DNA amplification reactions. Tne DNA polymerase mutants devoid of or substantially reduced in 3′→ 5′ exonuclease activity, devoid of or substantially reduced in 5′→ 3′ exonuclease activity, or containing a Phe^{67}→Tyr^{67} mutation are especially useful for DNA sequencing, DNA labeling, and DNA amplification reactions. Moreover, Tne polymerase mutants containing two or more of these properties are also especially useful for DNA sequencing, DNA labeling, on DNA amplification reactions. As is well known, sequencing reactions (dideoxy DNA sequencing and cycle DNA sequencing of plasmid DNA) require the use of DNA polymerases. Dideoxy-mediated sequencing involves the use of a chain-termination technique which uses a specific polymer for extension by DNA polymerase, a base-specific chain terminator and the use of polyacrylamide gels to separate the newly synthesized chain-terminated DNA molecules by size so that

polymerase of the present invention may be useful for incorporating αS nucleotides ([αS]dATP, [αS]dTTP, [αS]dCTP and [αS]dGTP) during sequencing (or labeling) reactions. For example, [α^{35}S]dATP, a commonly used detectably labeled nucleotide in sequencing reactions, is incorporated three times more efficiently with the Tne DNA polymerase of the present invention, than with Taq DNA polymerase. Thus, the enzyme of the present invention is particularly suited for sequencing or labeling DNA molecules with [α^{35}S]dNTPs.

Polymerase chain reaction (PCR), a well known DNA amplification technique, is a process by which DNA polymerase and deoxyribonucleoside triphosphates are used to amplify a target DNA template. In such PCR reactions, two primers, one complementary to the 3′ termini (or near the 3′-termini) of the first strand of the DNA molecule to be amplified, and a second primer complementary to the 3′ termini (or near the 3′-termini) of the second strand of the DNA molecule to be amplified, are hybridized to their respective DNA molecules. After hybridization, DNA polymerase, in the presence of deoxyribonucleoside triphosphates, allows the synthesis of a third DNA molecule complementary to the first strand and a fourth DNA molecule complementary to the second strand of the DNA molecule to be amplified. This synthesis results in two double stranded DNA molecules. Such double stranded DNA molecules may then be used as DNA templates for synthesis of additional DNA molecules by providing a DNA polymerase, primers, and deoxyribonucleoside triphosphates. As is well known, the additional synthesis is carried out by "cycling" the original reaction (with excess primers and deoxyribonucleoside triphosphates) allowing multiple denaturing and synthesis steps. Typically, denaturing of double stranded DNA molecules to form single stranded DNA templates is accomplished by high temperatures. The wild type and mutant *Thermotoga* DNA polymerases of the present invention are heat stable DNA polymerases, and thus will survive such thermal cycling during DNA amplification reactions. Thus, the wild type and mutant Tne DNA polymerases of the invention are ideally suited for PCR reactions, particularly where high temperatures are used to denature the DNA molecules during amplification.

Having now generally described the invention, the same will be more readily understood through reference to the following Examples which are provided by way of illustration, and are not intended to be limiting of the present invention, unless specified.

Example 1: Bacterial Strains And Growth Conditions

Thermotoga neapolitana DSM No. 5068 was grown under anaerobic conditions as described in the DSM catalog (addition of resazurin, Na_2S, and sulfur granules while sparging the media with nitrogen) at 85°C in an oil bath from 12 to 24 hours. The cells were harvested by filtering the broth through Whatman #1 filter paper. The supernatant was collected in an ice bath and then centrifuged in a refrigerated centrifuge at 8,000 rpms for twenty minutes. The cell paste was stored at -70°C prior to total genomic DNA isolation.

E. coli strains were grown in 2X LB broth base (Lennox L broth base: GIBCO/BRL) medium. Transformed cells were incubated in SOC (2% tryptone, 0.5% yeast extract, yeast 10 mM NaCl, 2.5 M KCl, 20mM glucose, 10mM $MgCl_2$, and 10mM $MgSO_4$ per liter) before plating. When appropriate antibiotic supplements were 20 mg/l tetracycline and 100 mg/l ampicillin. *E. coli* strain DH10B (Lorow *et al.*, *Focus* 12:19-20 (1990)) was used as host strain. Competent DH10B may be obtained from Life Technologies, Inc. (LTI) (Gaithersburg, MD).

Example 2: DNA Isolation

Thermotoga neapolitana chromosomal DNA was isolated from 1.1g of cells by suspending the cells in 2.5 ml TNE (50mM Tris-HCl, pH 8.0, 50mM NaCl, 10mM EDTA) and treated with 1% SDS for 10 minutes at 37°C. DNA was extracted with phenol by gently rocking the lysed cells overnight at 4°C. The next day, the lysed cells were extracted with chloroform:isoamyl alcohol. The resulting chromosomal DNA was further purified by centrifugation in a CsCl

fumes of chloroform:toluene (1:1) for 20 minutes and dried for 10 minutes at room temperature. The membranes were then treated at 95°C for 5 minutes to inactivate the endogenous *E. coli* enzymes. Surviving DNA polymerase activity was detected by submerging the membranes in 15 ml of polymerase reaction mix (50 mM Tris-HCl (pH 8.8), 1 mM $MgCl_2$, 3 mM β-mercaptoethanol, 10 μM dCTP, dGTP, dTTP, and 15 μCi of 3,000 Ci/mmol [α^{32}P]dATP) for 30 minutes at 65°C.

Using autoradiography, three colonies were identified that expressed a *Thermotoga neapolitana* DNA polymerase. The cells were grown in liquid culture and the protein extract was made by sonication. The presence of the cloned thermostable polymerase was confirmed by treatment at 90°C followed by measurement of DNA polymerase activity by incorporation of radioactive deoxyribonucleoside triphosphates into acid insoluble DNA. One of the clones, expressing Tne DNA polymerase, contained a plasmid designated pCP13-32 was used for further study.

Example 5: Subcloning of Tne DNA polymerase

Since the pCP13-32 clone expressing the Tne polymerase gene contains about 25 kb of *T. neapolitana* DNA, we attempted to subclone a smaller fragment of the Tne polymerase gene. The molecular weight of the Tne polymerase purified from *E. coli*/pCP13-32 was about *100* kd. Therefore, a 2.5-3.0 kb DNA fragment will be sufficient to code for full-length polymerase. A second round of *Sau*3A partial digestion similar to Example 3 was done using pCP13-32 DNA. In this case, a 3.5 kb region was cut out from the agarose gel, purified by Gene Clean (BIO 101, La Jolla, CA) and ligated into plasmid pSport 1 (Life Technologies, Inc.) which had been linearized with *Bam*HI and dephosphoylated with calf intestinal phosphatase. After ligation, DH10B was transformed and colonies were tested for DNA polymerase activity as described in Example 4. Several clones were identified that expressed Tne DNA polymerase. One of the clones (pSport-Tne) containing about 3 kb insert was further characterized. A restriction

fractions were dialyzed against 20 volumes of column buffer. The pooled fractions were applied to a Toso650Q column (Tosohaas). The column was washed to baseline OD_{280} and elution effected with a linear 10 column volume gradient of 25 mM Tris, pH 7.4, 8% glycerol, 0.5 mM EDTA, 10 mM KCl, 5 mM β-mercaptoethanol to the same buffer plus 650 mM KCl. Active fractions were pooled.

Example 7: Characterization of Purified Tne DNA Polymerase

1. Determination of the Molecular Weight of Thermotoga neapolitana DNA Polymerase

The molecular weight of 100 kilodaltons was determined by electrophoresis in a 12.5% SDS gel by the method of Laemmli, U.K., *Nature* (Lond.) 227:680-685 (1970). Proteins were detected by staining with Coomassie brilliant blue. A 10 kd protein ladder (Life Technologies, Inc.) was used as standard.

2. Method for Measuring Incorporation of [α^{35}S]-dATP Relative to ^{3}H-dATP

Incorporation of [αS]dATP was evaluated in a final volume of 500 μl of reaction mix, which was preincubated at 72°C for five minutes, containing either a [^{3}H]TTP nucleotide cocktail (100 μM each TTP, dATP, dCTP, dGTP with [^{3}H]TTP at 90.3 cpm/pmol), a nucleotide cocktail containing [αS]dATP as the only source of dATP (100 μM each [αS]dATP, dCTP, dGTP, TTP with [α^{35}S]dATP at 235 cpm/pmol), or a mixed cocktail (50 μM [αS]dATP, 50 μM dATP, 100 μM TTP, 100 μM dCTP, 100 μM dGTP with [^{35}αS] dATP at 118 cpm/pmol and [^{3}H]TTP at 45.2 cpm/pmol). The reaction was initiated by the addition of 0.3 units of *T. neapolitana* DNA polymerase or *T. aquaticus* DNA polymerase. At the times indicated a 25 μl aliquot was removed and quenched by addition of ice cold EDTA to a final concentration of 83 mM. 20 μl aliquots of

mM KCl, 10 mM $MgCl_2$, 10 mM dithiothreitol, and 100 μM [^{3}H]dGTP (132 cpm/pmole), or 100 mM KCl, 0.5 mM $MnCl_2$, and 200 μM [^{3}H]dGTP (107 cpm/pmole). Reaction mixtures also contained either 2.5 units of the Tth DNA polymerase (Perkin-Elmer) or 2.5 units of the Tne DNA polymerase. Incubations were at 45°C for 10 min followed by 75°C for 20 min.

The table shows the results of determining the relative levels of incorporation of Tne and Tth DNA polymerase with $(A)_n$:$(dT)_{20}$ and $(C)_n$:$(dG)_{12-18}$ in the presence of Mg^{++} and Mn^{++}. Tne DNA polymerase appears to be a better reverse transcriptase than Tth DNA polymerase under reaction conditions more specific for reverse transcriptase, i.e., in the presence of $(A)_n$:$(dT)_{20}$ with Mg^{++} and $(C)_n$:$(dG)_{12-18}$ with Mn^{++} or Mg^{++}.

DNA Polymerase Activity of Tth and Tne
DNA Polymerase with $(A)_n$:$(dT)_{20}$ and $(C)_n$:$(dG)_{12-18}$

Enzyme	DNA Polymerase Activity (pMoles Complementary [^{3}H]dNTP Incorporated)	
	$(A)_n$:$(dT)_{20}$	$(C)_n$:(dG)
	Mg^{++} Mn^{++}	Mg^{++} Mn^{++}
Tne	161.8 188.7	0.6 4.2
Tth	44.8 541.8	0 0.9

Example 9: Construction of Thermotoga Neapolitana 3'-to-5' Exonuclease Mutant

The amino acid sequence of portions of the Tne DNA polymerase was compared with other known DNA polymerases such as *E. coli* DNA polymerase 1, Taq DNA polymerase, T5 DNA polymerase, and T7 DNA polymerase to localize the regions of 3'-to-5' exonuclease activity, and the dNTP binding domains within the DNA polymerase. We have determined that one of the 3'-to-5' exonuclease domains based on the comparison of the amino acid sequences of

(3'→5'), was isolated. The presence of the mutant sequence was confirmed by the presence of the unique *Eco47*III site. The plasmid was then digested with *Sst*I and *Hind*III. The entire mutant polymerase gene (2.6 kb) was purified and cloned into *Sst*I and *Hind*III digested pTrc99 expression vector (Pharmacia, Sweden). The clones were selected in DH10B (LTI, Gaithersburg, MD). The resulting plasmid was designated pTrcTne35. See Figure 6B. This clone produced active heat stable DNA polymerase.

Example 10: Phenylalanine to Tyrosine Mutant

As discussed *supra*, the polymerase active site including the dNTP binding domain is usually present at the carboxyl terminal region of the polymerase. The preliminary and partial sequence of the Tne polymerase gene suggests that the amino acids that presumably contact and interact with the dNTPs are present within the 694 bases starting at the internal *Bam*HI site. *See* Figure 4. This conclusion is based on homology with a prototype polymerase *E. coli* polymerase 1. *See* Polisky et al., *J. Biol. Chem.* 265:14579-14591 (1990). The sequence of the carboxyl terminal portion of the polymerase gene is shown in Figure 5. Based upon this sequence, it is possible to compare the amino acid sequence within the O-helix for various polymerases:

		*		
Tne	63	KMVNFSIIYG	72	(SEQ ID NO. 9)
Pol I	758	KAINFGLIYG	767	(SEQ ID NO. 10)
T5	566	KAITFGILYG	575	(SEQ ID NO. 11)
T7	522	KTFIYGFLYG	531	(SEQ ID NO. 12)
Taq	663	KTINFGVLYG	672	(SEQ ID NO. 13)

It was shown that by replacing the phenylalanine residue of Taq DNA polymerases, (indicated as * above) the polymerase becomes non-discriminating against non-natural nucleotides such as dideoxynucleotides. See application Serial No. 08/525,087 entitled "Mutant DNA Polymerases and Use Thereof" of Deb K. Chatterjee, filed September 8, 1995, specifically incorporated herein by reference.

the smallest *Bam*HI fragment (0.8 kb) containing the $Phe^{67} \rightarrow Tyr^{67}$ mutation was purified and ligated to generate pUC-Tne35FY. The proper orientation and the presence of both mutations in the same plasmid was confirmed by *Eco*47III, *Hpa*I, and *Sph*I-*Hind*III restriction digests. See Figure 7.

The entire polymerase containing both mutations was subcloned as a SstI-HindIII fragment in pTrc99 to generate pTrcTne35FY in DH10B. The clone produced active heat stable polymerase.

Example 12: 3'-to- 5'Exonuclease, 5'- to-3' Exonuclease, and $Phe^{67} \rightarrow Tyr^{67}$ Triple Mutants

In most of the known polymerases, the 5'-to-3' exonuclease activity is present at the amino terminal region of the polymerase (Ollis, D.L., *et al.*, *Nature* 313, 762-766, 1985; Freemont, P.S., *et al.*, *Proteins* 1, 66-73, 1986; Joyce, C.M., Curr. Opin. Struct. Biol. 1, 123-129, 1991). There are some conserved amino acids that are implicated to be responsible for 5'-to-3' exonuclease activity (Gutman and Minton, *Nucl. Acids Res. 21*, 4406-4407, 1993). *See supra.* It is known that 5'-to-3' exonuclease domain is dispensable. The best known example is the Klenow fragment of *E. coli* Pol I. The Klenow fragment is a natural proteolytic fragment devoid of 5'-to-3' exonuclease activity (Joyce, C.M., *et al.*, *J. Biol. Chem.* 257, 1958-1964, 1990). In order to generate an equivalent mutant for Tne DNA polymerase devoid of 5'-to-3' exonuclease activity we exploited the presence of a unique *Sph*I site present 680 bases from the *Sst*I site. pUC-Tne35FY was digested with *Hind*III, filled-in with Klenow fragment to generate a blunt-end, and digested with *Sph*I. The 1.9 kb fragment was cloned into an expression vector pTTQ19 (Stark, M.J.R., *Gene* 51, 255-267, 1987) at the *Sph*I-*Sma*I sites and was introduced into DH10B. This cloning strategy generated an in-frame polymerase clone with an initiation codon for methionine from the vector. The resulting clone is devoid of 219 amino terminal amino acids of Tne DNA polymerase. This clone is designated as pTTQTne535FY. The clone produced active heat stable polymerase. No exonuclease activity could be

Example 14: Purification of the Mutant Polymerases

The purification of the mutant polymerases was done essentially as described in U.S. Patent Application Serial No. 08/370,190, filed January 9, 1995, entitled "Cloned DNA Polymerases for *Thermotoga neapolitana*," and as in Example 6, *supra*, with minor modifications. Specifically, 5 to 10 grams of cells expressing cloned mutant Tne DNA polymerase were lysed by sonication with a Heat Systems Ultrasonic, Inc. Model 375 machine in a sonication buffer comprising 50 mM Tris-HCl, pH 7.4; 8% glycerol; 5 mM 2-mercaptoethanol, 10 mM NaCl, 1 mM EDTA, and 0.5 mM PMSF. The sonication sample was heated at 75°C for 15 minutes. Following heat treatment, 200 mM NaCl and 0.4% PEI was added to remove nucleic acids. The extract was centrifuged for clarification. Ammonium sulfate was added to 48%, the pellet was resuspended in a column buffer consisting of 25 mM Tris-HCl, pH 7.4; 8% glycerol; 0.5% EDTA; 5 mM 2-mercaptoethanol; 10 mM KCl and loaded on a Heparin agarose column. The column was washed with 10 column volumes using the loading buffer and eluted with a 10 column volume buffer gradient from 10 mM to 1 M KCl. Fractions containing polymerase activity were pooled and dialyzed in column buffer as above with the pH adjusted to 7.8. The dialyzed pool of fractions were loaded onto a mono Q column. The column was washed and eluted as described above for the Heparin column. The active fractions are pooled and a unit assay was performed.

The unit assay reaction mixture contained 25 mM TAPS pH 9.3, 2 mM $MgCl_2$, 50 mM KCl, 1 mM DTT, 0.2 mM dNTPs, 500 µg/ml DNAse I treated salmon sperm DNA, 21 mCi/ml [αP^{32}] dCTP and various amounts of polymerase in a final volume of 50 ml. After 10 minutes incubation at 70°C, 10 ml of 0.5 M EDTA was added to the tube. TCA precipitable counts were measured in GF/C filters using 40 ml of the reaction mixture.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:LIFE TECHNOLOGIES, INC.

(ii) TITLE OF INVENTION: Cloned DNA Polymerases from Thermotoga Neapolitana and Mutants Thereof

(iii) NUMBER OF SEQUENCES: 3

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: STERNE, KESSLER, GOLDSTEIN & FOX
(B) STREET: 1100 New York Avenue, N.W.
(C) CITY: Washington
(D) STATE: DC
(E) COUNTRY: USA
(F) ZIP: 20005

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: TO BE ASSIGNED
(B) FILING DATE: 02-OCT-1995
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/316,423
(B) FILING DATE: 30-SEP-1994

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/370,190
(B) FILING DATE: 09-JAN-1995

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Esmond, Robert W.
(B) REGISTRATION NUMBER: 32,893
(C) REFERENCE/DOCKET NUMBER: 0942.280PC02

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 202-371-2600
(B) TELEFAX: 202-371-2540

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

ATGAAATACA GAGGATACGA CAAGAGAAAA CTACTTCCGA TATTGAAAGA ACTGGAGTTT 840

GCTTCCATCA TGAAGGAACT TCAACTGTAC GAAGAAGCAG AACCCACCGG ATACGAAATC 900

GTGAAGGATC ATAAGACCTT CGAAGATCTC ATCGAAAAGC TGAAGGAGGT TCCATCTTTT 960

GCCCTGGACC TTGAAACGTC CTCCTTGGAC CCGTTCAACT GTGAGATAGT CGGCATCTCC 1020

GTGTCGTTCA AACCGAAAAC AGCTTATTAC ATTCCACTTC ATCACAGAAA CGCCCACAAT 1080

CTTGATGAAA CACTGGTGCT GTCGAAGTTG AAAGAGATCC TCGAAGACCC GTCTTCGAAG 1140

ATTGTGGGTC AGAACCTGAA GTACGACTAC AAGGTTCTTA TGGTAAAGGG TATATCGCCA 1200

GTTTATCCGC ATTTTGACAC GATGATAGCT GCATATTTGC TGGAGCCAAA CGAGAAAAAA 1260

TTCAATCTCG AAGATCTGTC TTTGAAATTT CTCGGATACA AAATGACGTC 1310

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 436 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met Ala Arg Leu Phe Leu Phe Asp Gly Thr Ala Leu Ala Tyr Arg Ala
1 5 10 15

Tyr Tyr Ala Leu Asp Arg Ser Leu Ser Thr Ser Thr Gly Ile Pro Thr
20 25 30

Asn Ala Val Tyr Gly Val Ala Arg Met Leu Val Lys Phe Ile Lys Glu
35 40 45

His Ile Ile Pro Glu Lys Asp Tyr Ala Ala Val Ala Phe Asp Lys Lys
50 55 60

Ala Ala Thr Phe Arg His Lys Leu Leu Val Ser Asp Lys Ala Gln Arg
65 70 75 80

Pro Lys Thr Pro Ala Leu Leu Val Gln Gln Leu Pro Tyr Ile Lys Arg
85 90 95

Leu Ile Glu Ala Leu Gly Phe Lys Val Leu Glu Leu Glu Gly Tyr Glu
100 105 110

Val Tyr Pro His Phe Asp Thr Met Ile Ala Ala Tyr Leu Leu Glu Pro
405 410 415

Asn Glu Lys Lys Phe Asn Leu Glu Asp Leu Ser Leu Lys Phe Leu Gly
420 425 430

Tyr Lys Met Thr
435

10. The recombinant host of claim 9, wherein said gene is obtained from *Thermotoga neapolitana* DSM 5068.

11. The recombinant host of claim 8, wherein said host is prokaryotic.

12. The recombinant host of claim 11, wherein said host is *E. coli*.

13. A method of producing a Tne DNA polymerase having a molecular weight of about 100 kilodaltons, said method comprising:

(a) culturing a cellular host comprising a gene encoding said DNA polymerase;

(b) expressing said gene; and

(c) isolating said DNA polymerase from said host.

14. The method of claim 13, wherein said host is a eukaryotic host.

15. The method of claim 13, wherein said host is a prokaryotic host.

16. The method of claim 15, wherein said prokaryotic host is *E. coli*.

17. A method of synthesizing a double-stranded DNA molecule comprising:

(a) hybridizing a primer to a first DNA molecule; and

(b) incubating said DNA molecule of step (a) in the presence of one or more deoxyribonucleoside triphosphates and Tne DNA polymerase having a molecular weight of about 100 kilodaltons, under conditions sufficient to synthesize a second DNA molecule complementary to all or a portion of said first DNA molecule.

18. The method of claim 17, wherein said DNA polymerase is isolated from *Thermotoga neapolitana*.

(c) incubating the mixture of step (b) under conditions sufficient to synthesize a random population of DNA molecules complementary to said first DNA molecule,
wherein said synthesized DNA molecules are shorter in length than said first DNA molecule and wherein said synthesized DNA molecules comprise a terminator nucleotide at their 5′ termini; and (d) separating said synthesized DNA molecules by size so that at least a part of the nucleotide sequence of said first DNA molecule can be determined.

28. The method of claim 27, wherein said terminator nucleotide is ddTTP.

29. The method of claim 27, wherein said terminator nucleotide is ddATP.

30. The method of claim 27, wherein said terminator nucleotide is ddGTP.

31. The method of claim 27, wherein said terminator nucleotide is ddCTP.

32. The method of claim 27, wherein said DNA polymerase is isolated from *Thermotoga neapolitana*.

33. The method of claim 32, wherein said DNA polymerase is isolated from *Thermotoga neapolitana* DSM 5068.

34. The method of claim 27, wherein said DNA polymerase is isolated from a recombinant host expressing a gene encoding said DNA polymerase.

(a) a first container means comprising a Tne DNA polymerase having a molecular weight of about 100 kilodaltons;

(b) a second container means comprising one or more dideoxyribonucleoside triphosphates; and

(c) a third container means comprising one or more deoxyribonucleoside triphosphates.

42. The kit of claim 41, wherein said DNA polymerase is isolated from *Thermotoga neapolitana*.

43. The kit of claim 42, wherein said DNA polymerase is isolated from *Thermotoga neapolitana* DSM 5068.

44. The kit of claim 41, wherein said DNA polymerase is isolated from a recombinant host expressing a gene encoding said DNA polymerase.

45. A kit for amplifying a DNA molecule, comprising:

(a) a first container means comprising a Tne DNA polymerase having a molecular weight of about 100 kilodaltons; and

(b) a second container means comprising one or more deoxyribonucleoside triphosphates.

46. The kit of claim 45, wherein said DNA polymerase is isolated from *Thermotoga neapolitana*.

47. The kit of claim 46, wherein said DNA polymerase is isolated from *Thermotoga neapolitana* DSM 5068.

48. The kit of claim 45, wherein said DNA polymerase is isolated from a recombinant host expressing a gene encoding said DNA polymerase.

DNA polymerase; and (3) a third mutation in the O helix of said DNA polymerase resulting in said DNA polymerase becoming non-discriminating against dideoxynucleotides, or fragments thereof.

56. The isolated DNA molecule as claimed in claim 55, wherein said DNA molecule is selected from the group consisting of pTrcTne35, pTrcTneFY, pTrcTne35FY, and pTTQTne535FY.

57. The isolated DNA molecule as claimed in claim 55, wherein said DNA molecule further comprises expression control elements.

58. The isolated DNA molecule as claimed in claim 57, wherein said expression control elements comprise an inducible promoter selected from the group consisting of λP_L promoter, a tac promoter, a trp promoter, and a trc promoter.

59. A recombinant host comprising a DNA sequence encoding a mutant *Thermotoga neapolitana* DNA polymerase having at least one mutation selected from the group consisting of (1) a first mutation that substantially reduces or eliminates 3′→5′ exonuclease activity of said DNA polymerase; (2) a second mutation that substantially reduces or eliminates 5′→3′ exonuclease activity of said DNA polymerase; and (3) a third mutation in the O helix of said DNA polymerase resulting in said DNA polymerase becoming non-discriminating against dideoxynucleotides, or fragments thereof.

60. A method of producing a Tne DNA polymerase, said method comprising:

(a) culturing a cellular host comprising a gene encoding a mutant *Thermotoga neapolitana* DNA polymerase having at least one mutation selected from the group consisting of (1) a first mutation that substantially reduces or eliminates 3′→5′ exonuclease activity of said DNA polymerase; (2) a second

dUTP, ddATP, ddCTP, ddGTP, ddITP, ddTTP, [αS]dATP, [αS]dTTP, [αS]dGTP, and [αS]dCTP.

64. The method of synthesizing a double-stranded DNA molecule as claimed in claim 63, wherein one or more of said deoxyribonucleoside triphosphates are detectably labelled.

65. The method of synthesizing a double-stranded DNA molecule as claimed in claim 64, wherein said label is selected from the group consisting of a radioactive isotape, a fluorescent label, a chemiluminessant label, a bioluminescent label, and an enyzme label.

66. A method of sequencing a DNA molecule, comprising:

(a) hybridizing a primer to a first DNA molecule;

(b) contacting said DNA molecule of step (a) with deoxyribonucleoside triphosphates, a mutant *Thermotoga neapolitana* DNA polymerase, and a terminator nucleotide;

(c) incubating the mixture of step (b) under conditions sufficient to synthesize a random population of DNA molecules complementary to said first DNA molecule;

wherein said synthesized DNA molecules are shorter in length than said first DNA molecule and wherein said synthesized DNA molecules comprise a terminator nucleotide at their 5′ termini; and

(d) separating said synthesized DNA molecules by size so that at least a part of the nucleotide sequence of said first DNA molecule can be determined.

wherein said mutant *Thermotoga neapolitana* DNA polymerase has at least one mutation selected from the group consisting of (1) a first mutation that substantially reduces or eliminates 3′→5′ exonuclease activity of said DNA polymerase; (2) a second mutation that substantially reduces or eliminates 5′→3′ exonuclease activity of said DNA polymerase; and (3) a third mutation in the O

(a) a first container means comprising a mutant *Thermotoga neapolitana* DNA polymerase;

(b) a second container means comprising one or more dideoxyribonucleoside triphosphates; and

(c) a third container means comprising one or more deoxyribonucleoside triphosphates,

wherein:

said mutant *Thermotoga neapolitana* DNA polymerase has at least one mutation selected from the group consisting of (1) a first mutation that substantially reduces or eliminates 3′→5′ exonuclease activity of said DNA polymerase; (2) a second mutation that substantially reduces or eliminates 5′→3′ exonuclease activity of said DNA polymerase; and (3) a third mutation in the O helix of said DNA polymerase resulting in said DNA polymerase becoming non-discriminating against dideoxynucleotides.

70. A kit for amplifying a DNA molecule, comprising:

(a) a first container means comprising a mutant *Thermotoga neapolitana* DNA polymerase; and

(b) a second container means comprising one or more deoxyribonucleoside triphosphates,

wherein:

said mutant *Thermotoga neapolitana* DNA polymerase has at least one mutation selected from the group consisting of (1) a first mutation that substantially reduces or eliminates 3′→5′ exonuclease activity of said DNA polymerase; (2) a second mutation that substantially reduces or eliminates 5′→3′ exonuclease activity of said DNA polymerase; and (3) a third mutation in the O helix of said DNA polymerase resulting in said DNA polymerase becoming non-discriminating against dideoxynucleotides.

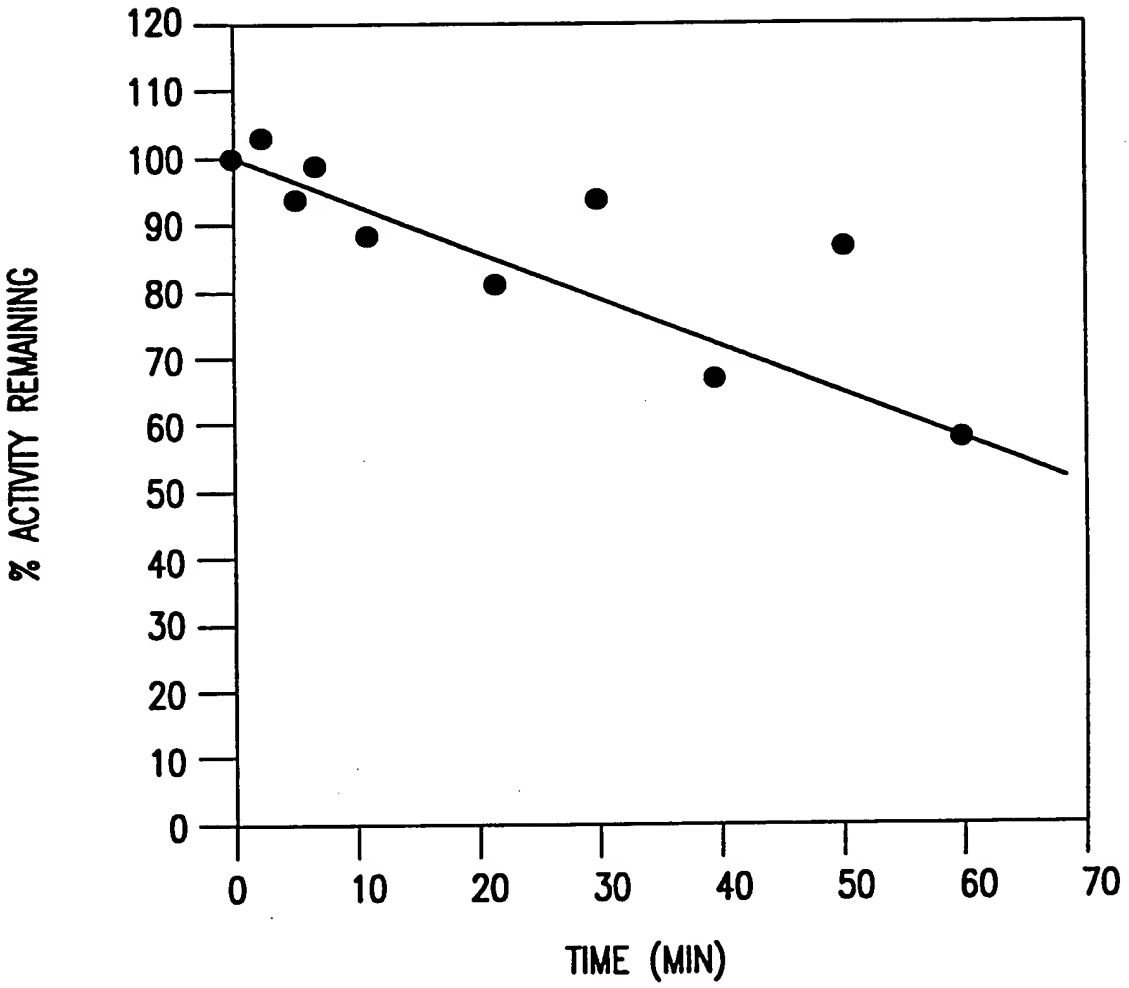

% ACTIVITY REMAINING
120
110
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
TIME (MIN)

FIG.1

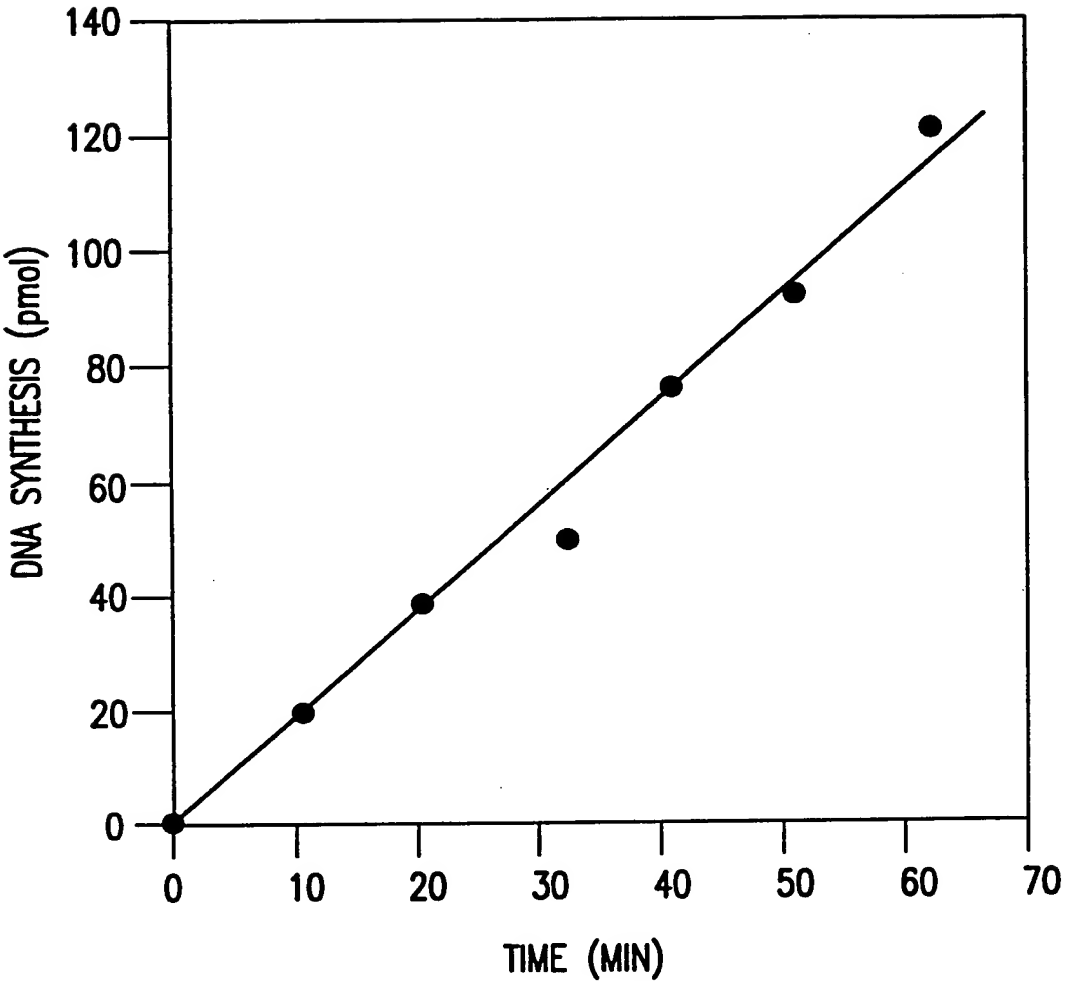

140
120
100
80
60
40
20
0
DNA SYNTHESIS (pmol)
0
10
20
30
40
50
60
70
TIME (MIN)

FIG.2

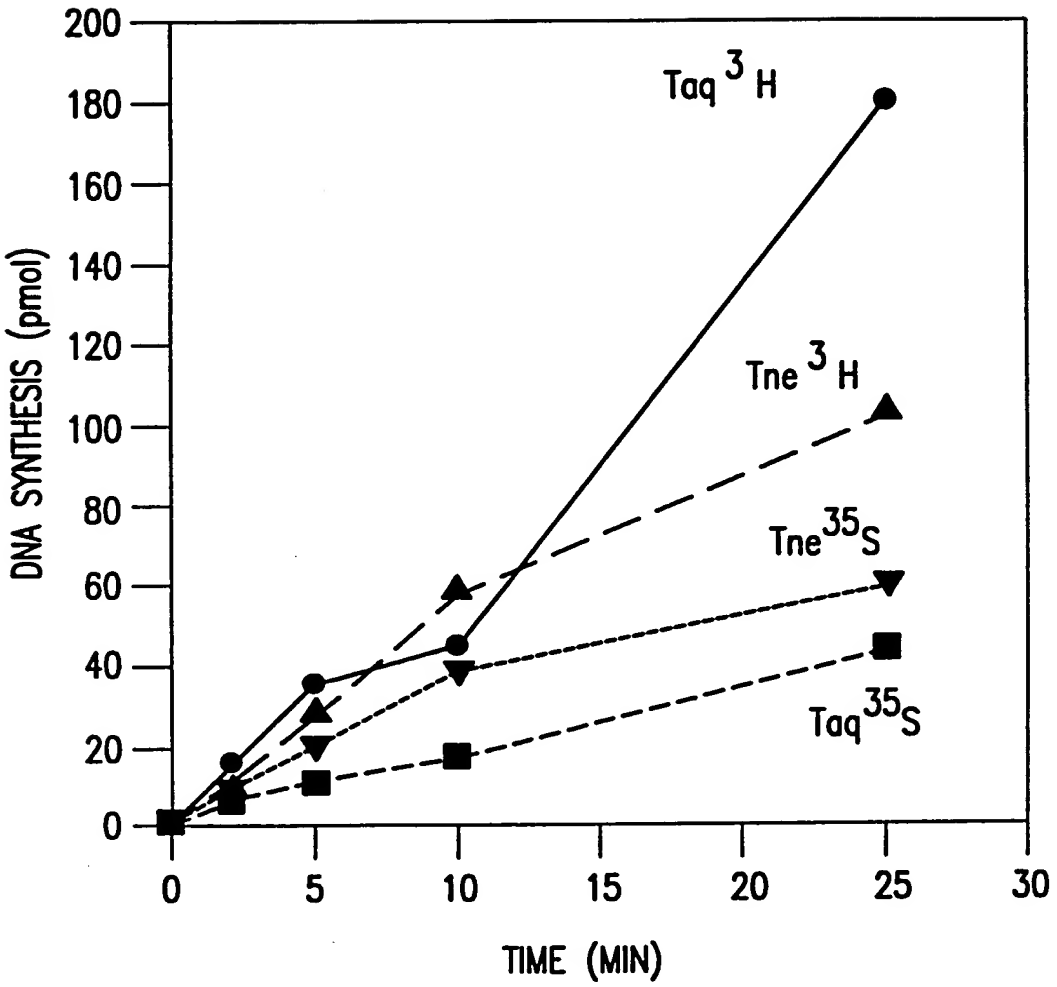

200
180
160
140
120
100
80
60
40
20
0
DNA SYNTHESIS (pmol)
0
5
10
15
20
25
30
TIME (MIN)
Taq 3 H
Tne 3 H
Tne35S
Taq35S

FIG.3

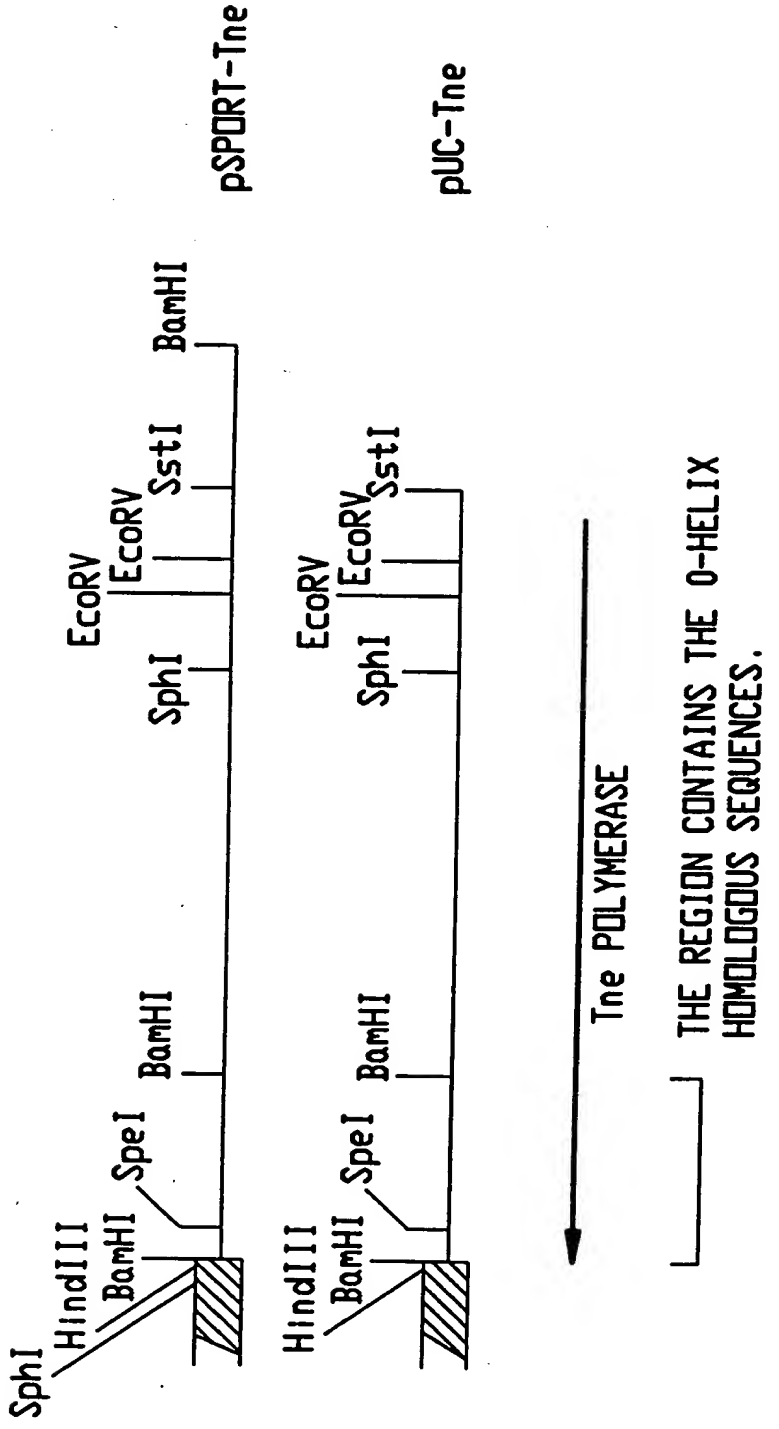

pSPORT-Tne
pUC-Tne
SphI
HindIII
BamHI
SpeI
BamHI
SphI
EcoRV
EcoRV
SstI
BamHI
HindIII
BamHI
SpeI
BamHI
SphI
EcoRV
EcoRV
SstI
Tne POLYMERASE
THE REGION CONTAINS THE O-HELIX
HOMOLOGOUS SEQUENCES.

FIG.4

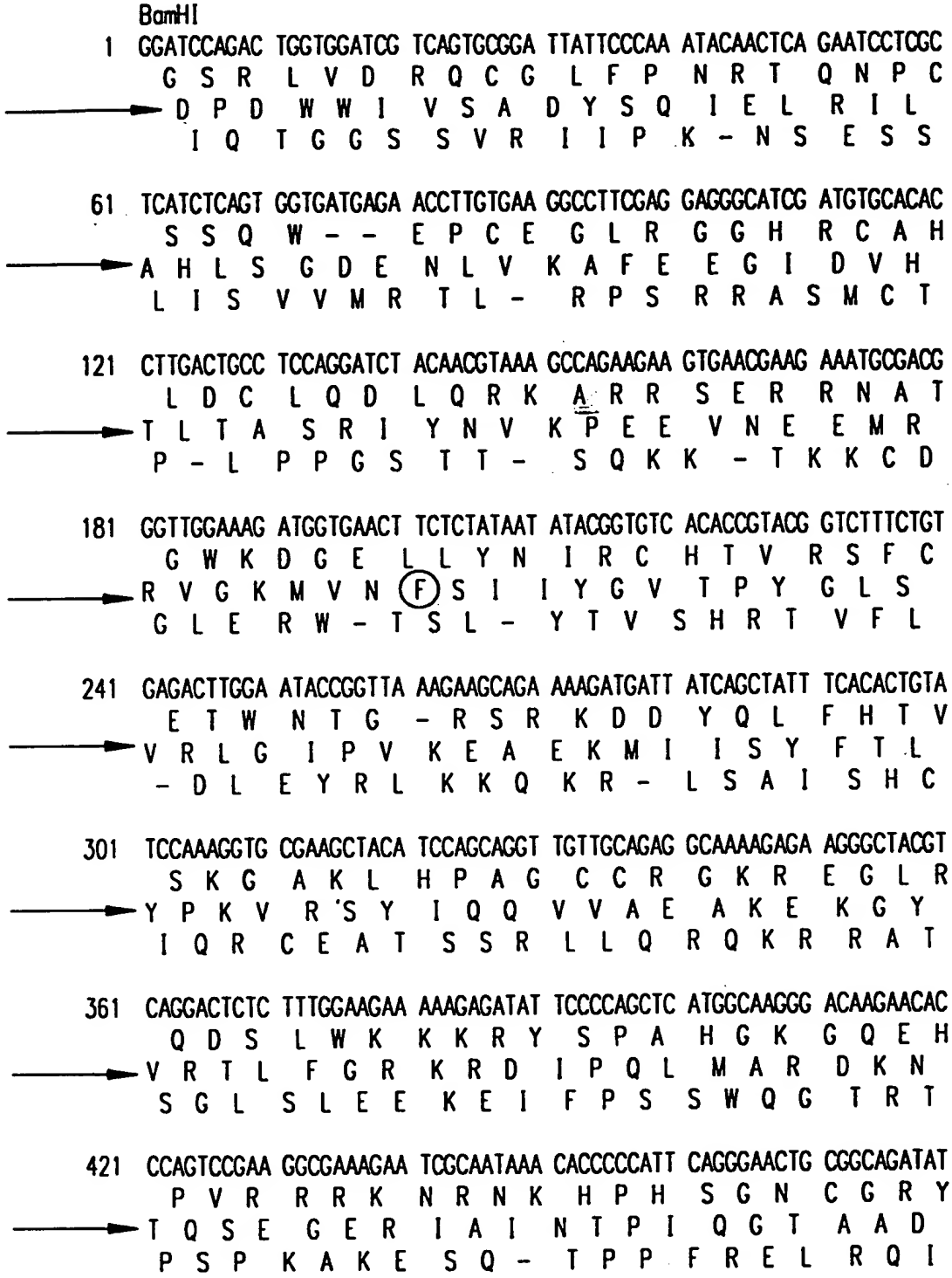

BamHI
1 GGATCCAGAC TGGTGGATCG TCAGTGCGGA TTATTCCCAA ATACAACTCA GAATCCTCGC
G S R L V D R Q C G L F P N R T Q N P C
D P D W W I V S A D Y S Q I E L R I L
I Q T G G S S V R I I P K - N S E S S
61 TCATCTCAGT GGTGATGAGA ACCTTGTGAA GGCCTTCGAG GAGGGCATCG ATGTGCACAC
S S Q W - - E P C E G L R G G H R C A H
A H L S G D E N L V K A F E E G I D V H
L I S V V M R T L - R P S R R A S M C T
121 CTTGACTGCC TCCAGGATCT ACAACGTAAA GCCAGAAGAA GTGAACGAAG AAATGCGACG
L D C L Q D L Q R K A R R S E R R N A T
T L T A S R I Y N V K P E E V N E E M R
P - L P P G S T T - S Q K K - T K K C D
181 GGTTGGAAAG ATGGTGAACT TCTCTATAAT ATACGGTGTC ACACCGTACG GTCTTTCTGT
G W K D G E L L Y N I R C H T V R S F C
R V G K M V N F S I I Y G V T P Y G L S
G L E R W - T S L - Y T V S H R T V F L
241 GAGACTTGGA ATACCGGTTA AAGAAGCAGA AAAGATGATT ATCAGCTATT TCACACTGTA
E T W N T G - R S R K D D Y Q L F H T V
V R L G I P V K E A E K M I I S Y F T L
- D L E Y R L K K Q K R - L S A I S H C
301 TCCAAAGGTG CGAAGCTACA TCCAGCAGGT TGTTGCAGAG GCAAAAGAGA AGGGCTACGT
S K G A K L H P A G C C R G K R E G L R
Y P K V R S Y I Q Q V V A E A K E K G Y
I Q R C E A T S S R L L Q R Q K R R A T
361 CAGGACTCTC TTTGGAAGAA AAAGAGATAT TCCCCAGCTC ATGGCAAGGG ACAAGAACAC
Q D S L W K K K R Y S P A H G K G Q E H
V R T L F G R K R D I P Q L M A R D K N
S G L S L E E K E I F P S S W Q G T R T
421 CCAGTCCGAA GGCGAAAGAA TCGCAATAAA CACCCCCATT CAGGGAACTG CGGCAGATAT
P V R R R K N R N K H P H S G N C G R Y
T Q S E G E R I A I N T P I Q G T A A D
P S P K A K E S Q - T P P F R E L R Q I

FIG.5A

```
481  AATAAAATTG GCTATGATAG ATATAGACGA GGAGCTGAGA AAAAGAAACA TGAAATCCAG
       N K I  G Y D  R Y R R  G A E  K K K  H E I  Q
     I I K L  A M I  D I D  E E L R  K R N  M K S
       - - N  W L - -  I - T  R S -  E K E T  - N P

541  AATGATCATT CAGGTTCATG ACGAACTGGT CTTCGAGGTT CCCGATGAGG AAAAAGAAGA
       N D H  S G S  - R T G  L R G  S R -  G K R R
     R M I I  Q V H  D E L  V F E V  P D E  E K E
       E - S  F R F M  T N W  S S R  F P M R  K K K

601  ACTAGTTGAT CTGGTGAAGA ACAAAATGAC AAATGTGGTG AAACTCTCTG TGCCTCTTGA
       T S -  S G E  E Q N D  K C G  E T L  C A S -
     E L V D  L V K  N K M  T N V V  K L S  V P L
       N - L  I W - R  T K -  Q M W  - N S L  C L L

661  GGTTGACATA AGGATGGGAA AAAGCTGGTC TTGA
       G - H  K H R  K K L V  L
     E V D I  S I G  K S W S  -
       R L T  - A S E  K A G L
```

FIG.5B

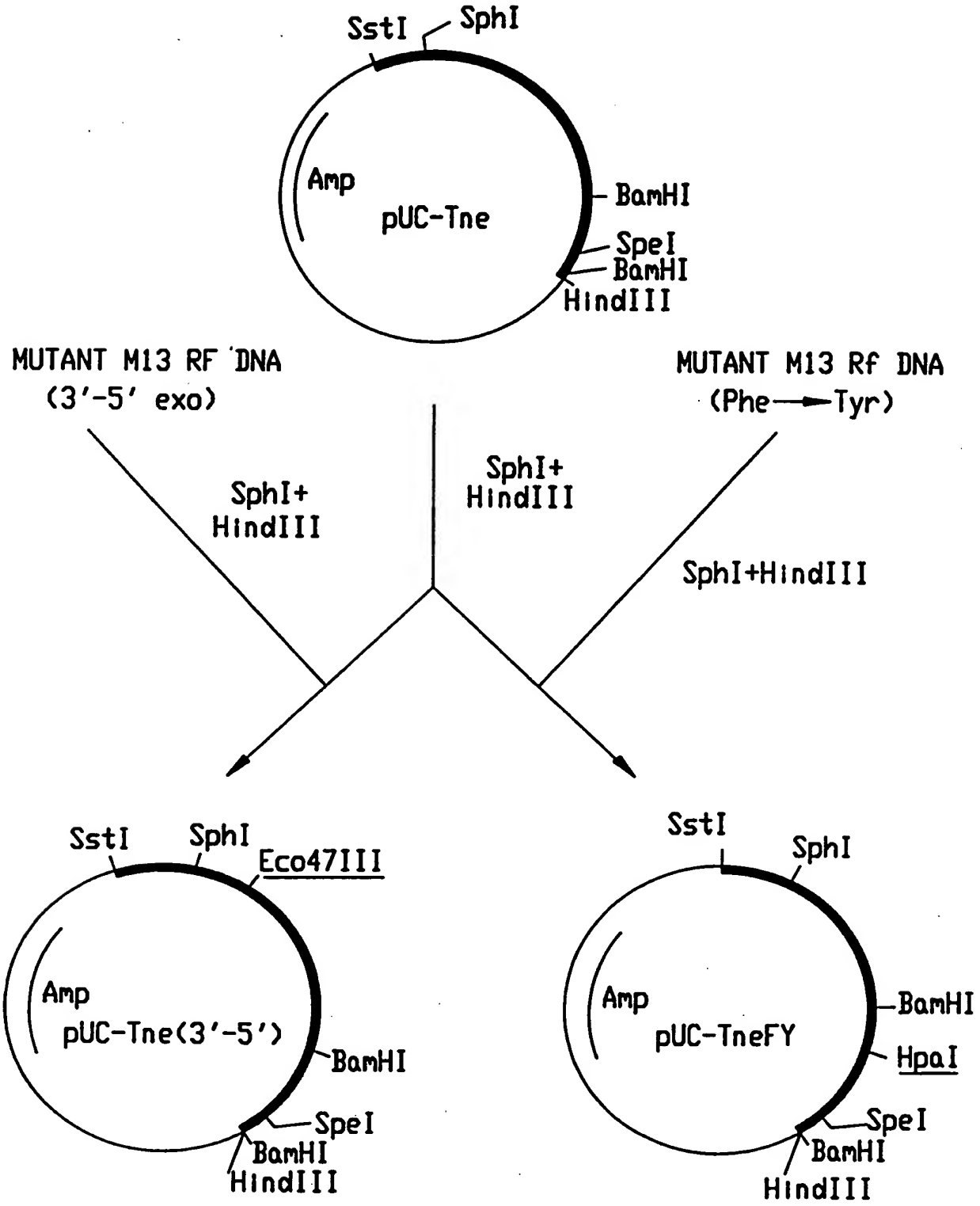

SstI
SphI
Amp
pUC-Tne
BamHI
SpeI
BamHI
HindIII
MUTANT M13 RF DNA
(3'-5' exo)
MUTANT M13 Rf DNA
(Phe→Tyr)
SphI+
HindIII
SphI+
HindIII
SphI+HindIII
SstI
SphI
Eco47III
Amp
pUC-Tne(3'-5')
BamHI
SpeI
BamHI
HindIII
SstI
SphI
Amp
pUC-TneFY
BamHI
HpaI
SpeI
BamHI
HindIII

FIG.6A

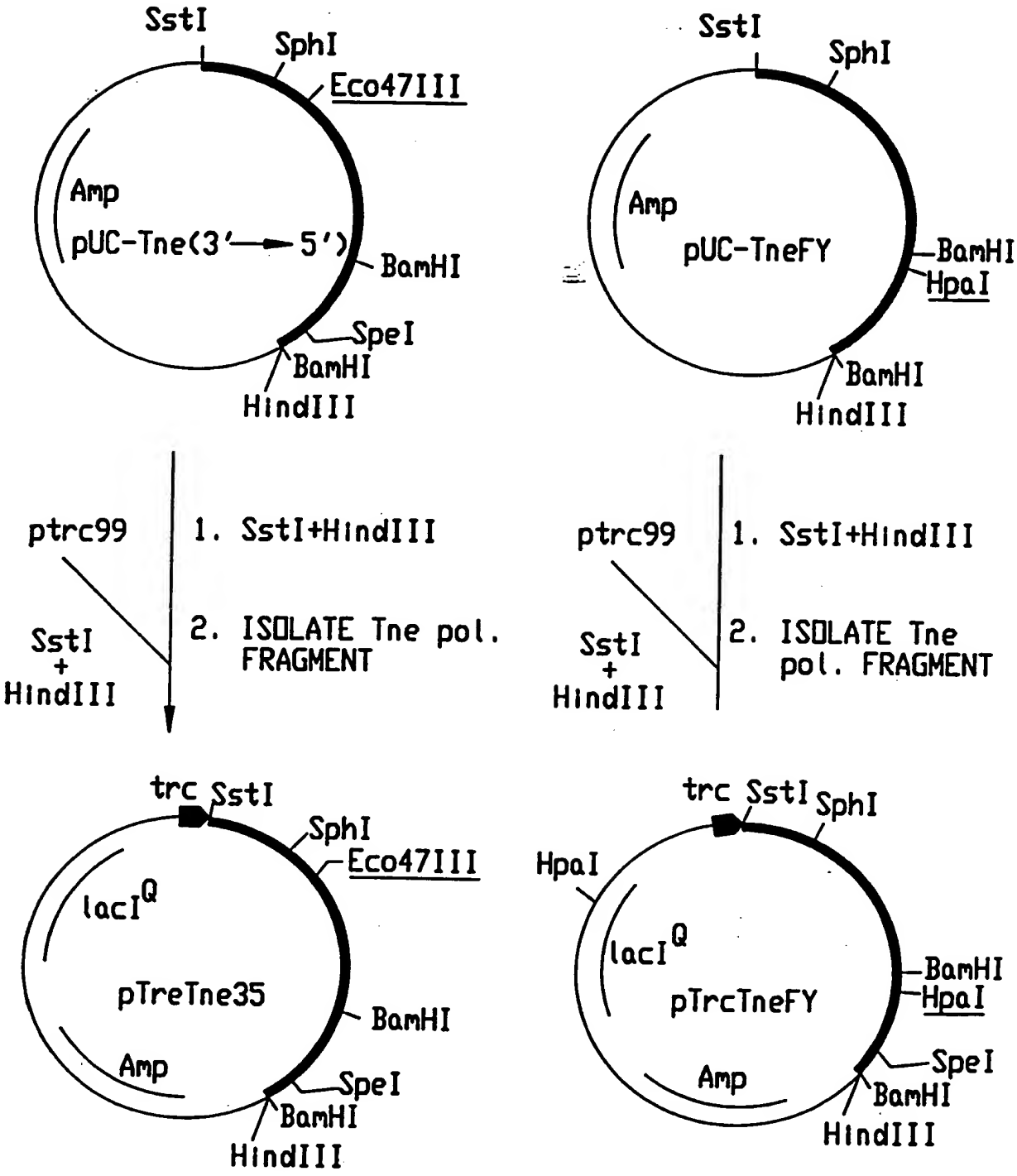

SstI
SphI
Eco47III
Amp
pUC-Tne(3'→5')
BamHI
SpeI
BamHI
HindIII
ptrc99
SstI
+
HindIII
1. SstI+HindIII
2. ISOLATE Tne pol. FRAGMENT
trc
SstI
SphI
Eco47III
lacI^Q
pTreTne35
BamHI
Amp
SpeI
BamHI
HindIII
SstI
SphI
Amp
pUC-TneFY
BamHI
HpaI
BamHI
HindIII
ptrc99
SstI
+
HindIII
1. SstI+HindIII
2. ISOLATE Tne pol. FRAGMENT
trc
SstI
SphI
HpaI
lacI^Q
pTrcTneFY
BamHI
HpaI
SpeI
Amp
BamHI
HindIII

FIG.6B

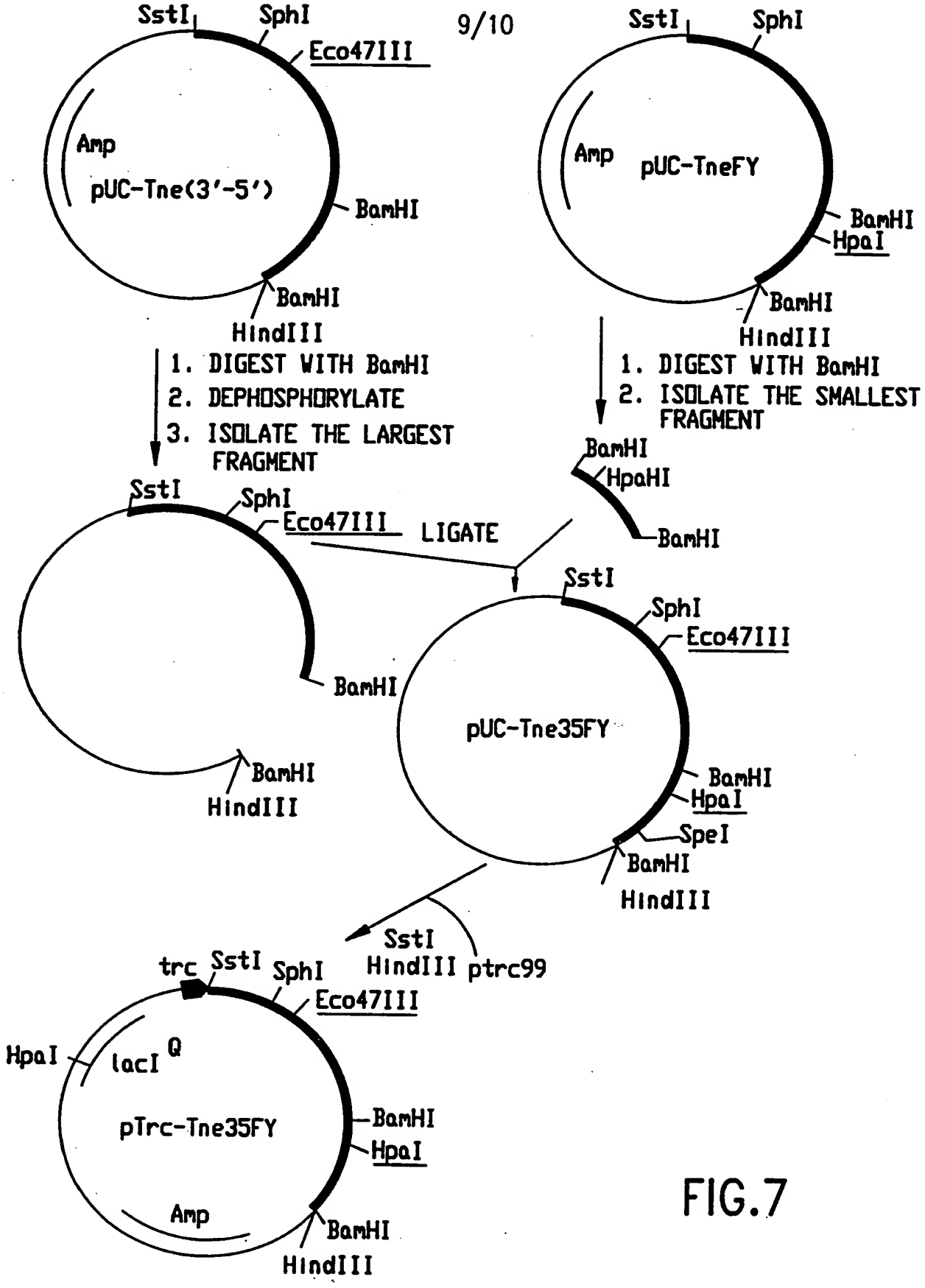

SstI
SphI
Eco47III
Amp
pUC-Tne(3'-5')
BamHI
BamHI
HindIII
1. DIGEST WITH BamHI
2. DEPHOSPHORYLATE
3. ISOLATE THE LARGEST FRAGMENT
SstI
SphI
Eco47III
BamHI
BamHI
HindIII
LIGATE
SstI
SphI
Eco47III
Amp
pUC-TneFY
BamHI
HpaI
BamHI
HindIII
1. DIGEST WITH BamHI
2. ISOLATE THE SMALLEST FRAGMENT
BamHI
HpaHI
BamHI
SstI
SphI
Eco47III
pUC-Tne35FY
BamHI
HpaI
SpeI
BamHI
HindIII
SstI
HindIII
ptrc99
trc
SstI
SphI
Eco47III
HpaI
lacI Q
pTrc-Tne35FY
BamHI
HpaI
Amp
BamHI
HindIII

FIG.7

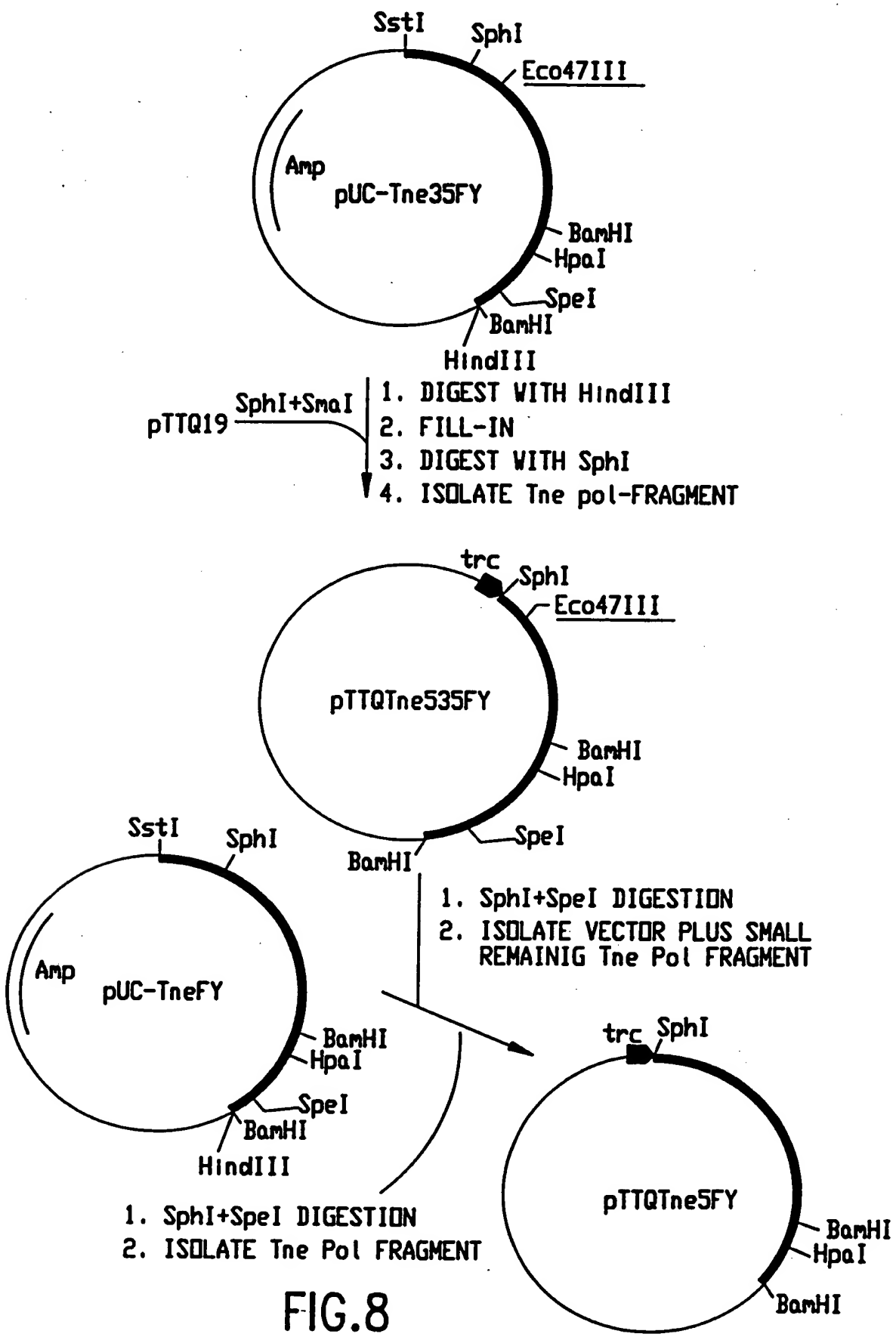

SstI
SphI
Eco47III
Amp
pUC-Tne35FY
BamHI
HpaI
SpeI
BamHI
HindIII
pTTQ19
SphI+SmaI
1. DIGEST WITH HindIII
2. FILL-IN
3. DIGEST WITH SphI
4. ISOLATE Tne pol-FRAGMENT
trc
SphI
Eco47III
pTTQTne535FY
BamHI
HpaI
SpeI
BamHI
SstI
SphI
Amp
pUC-TneFY
BamHI
HpaI
SpeI
BamHI
HindIII
1. SphI+SpeI DIGESTION
2. ISOLATE VECTOR PLUS SMALL REMAINIG Tne Pol FRAGMENT
trc
SphI
pTTQTne5FY
BamHI
HpaI
BamHI
1. SphI+SpeI DIGESTION
2. ISOLATE Tne Pol FRAGMENT

FIG.8

INTERNATIONAL SEARCH REPORT

Internal Application No
PCT/US 95/12358

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/54. C12N15/70 C12N9/12 C12N1/21 C12Q1/68
//(C12N1/21,C12R1:19)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ARCHIVES OF MICROBIOLOGY, vol. 150, no. 1, 1988 SPRINGER, BERLIN, BRD, pages 103-104, H.W. JANNASCH ET AL. 'Thermotoga neapolitana sp. nov. of the extremely thermophilic, eubacterial genus Thermotoga' see the whole document --- -/--	1-70

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 February 1996	14.03.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Hornig, H

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Internal Application No
PCT/US 95/12358

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WO-A-9206202	16-04-92	AU-B-	663474	12-10-95
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		AU-B-	8907791	28-04-92
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		EP-A-	0550687	14-07-93
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		DE-T-	655506	28-09-95